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Prolonged E55+ Retrovirus Expression in Aged Mice Is Associated with a Decline in the Anti-Virus Immune Response

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E55+ murine leukemia retrovirus (E55+MuLV) infection of young and aged C57BL/6 (B6) mice was used to investigate the relationship between increased incidences of infection and decreased immune responsiveness of elderly individuals. Young mice decreased E55+MuLV burden to below detectable levels by 8 weeks postinfection (p.i.). In contrast, virus burden in aged mice did not reach undetectable levels until 20 weeks p.i. A significant T cell proliferative response to E55+MuLV was detected from 2 to 12 weeks p.i. in young mice, but was never observed in aged mice. Both age groups demonstrated significant E55+MuLV-specific T-cell-mediated cytotoxic responses at 3 and 4 weeks p.i. and virus neutralizing antibody titers at 2, 4, 8, and 12 weeks p.i. In both cases, responses were consistently higher in young mice ($P < 0.04$ and $P < 0.02$, respectively). These results demonstrate that the observed delay in E55+MuLV clearance by aged mice is associated with an age-related decrease in the immune response to the virus.

Key Words: aging; retrovirus; $CD4^+$ T cell; $CD8^+$ T cell; neutralizing antibody.

INTRODUCTION

Epidemiologic data show that aging is associated with an increased incidence of virus infections. In addition, there is increased morbidity and mortality among the elderly as a result of these infections (Felser and Raff, 1983). For example, age is an important predictor of progression of HIV disease. Older patients infected with HIV not only develop AIDS faster than younger patients, they die more quickly after developing an AIDS-defining illness (Ship *et al.,* 1991; Adler and Nagel, 1994; Adler *et al.,* 1997). Although it has been shown that aging is associated with alterations in both T-cell-mediated immunity (Kirschmann and Murasko, 1992; Goonewardene and Murasko, 1994; Chakravarti and Abraham, 1999; Pawelec *et al.,* 1999) and humoral immunity (LeMaoult *et al.,* 1997; Song *et al.,* 1997), few studies have examined directly the relationship between decreased immune responsiveness and diminished control of virus infections in the aged.

E55+ murine leukemia virus (E55+MuLV) is a replication-competent murine retrovirus that causes leukemia in susceptible strains of mice (BALB) after a long latent period (Tumas *et al.,* 1993a). In contrast, infection of resistant strains of mice (C57BL) fails to progress to leukemia even though E55+MuLV establishes a persistent infection in both strains of mice (Avidan *et al.,* 1995). Infection of both resistant and susceptible mice is characterized by an acute phase and a persistent phase. The acute phase is characterized by the presence of large numbers of virus-infected cells in spleen and bone marrow during the first 2–4 weeks postinfection (p.i.). The number of virus-infected cells decreases to undetectable levels by 8 weeks p.i. due to the generation of an anti-virus immune response. The anti-virus immune response has been demonstrated to play an important role in controlling virus infection during this acute phase because mice irradiated sublethally prior to inoculation with E55+MuLV show no decrease in virus burden (Avidan *et al.,* 1995; Panoutsakopoulou *et al.,* 1998). However, despite the immune response, the virus is not eradicated with the infected cells sequestered in the lymphoid tissue (Tumas *et al.,* 1993b; Avidan *et al.,* 1995). During the persistent phase, BALB strains of mice demonstrate an increase in virus-infected cells at about 5–8 months p.i. that correlates with the development of leukemia. In contrast, no increase in infected cells occurs in C57BL strains of mice (Tumas *et al.,* 1993b; Avidan *et al.,* 1995; Panoutsakopoulou *et al.,* 1998).

Due to the importance of the anti-virus immune response in the control of E55+MuLV burden during the acute phase of infection, we utilized this model to directly test the hypothesis that decreased immune responsiveness with aging is associated with impaired virus clearance during the acute phase of infection. The present studies demonstrate that, unlike young C57BL/6 (B6) mice that decreased E55+MuLV burden by 8 weeks p.i.,

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TABLE 1

E55+ Virus Production in Young and Aged C57BL/6 Mice[a]

Age (months)	Weeks postinfection				
	2	4	8	12	20
6	1×10^3 (<10–3×10^3)	1×10^5 (4×10^3–5×10^5)	<10	<10	ND
22	1.3×10^3 (2×10^2–3×10^3)	8.3×10^4 (1×10^3–3×10^5)	4.9×10^5 (2×10^2–2×10^6)	2×10^3 (2×10^1–1×10^4)	<10

Note. Mean virus titers of 6–8 mice/time point expressed as FFU/ml of 10% spleen homogenate. Numbers in parentheses are the range of results. ND, not done.

[a] The level of infectious virus in the spleen of B6 mice was quantified by FFA at various times postinfection.

aged B6 mice showed a delay in virus clearance. This delay in virus clearance in aged mice was associated with the lack of a T cell proliferative response, a significantly lower cytotoxic T cell response to E55+MuLV, and significantly lower virus neutralizing antibody titers in aged mice than in young mice. These results suggest that the observed difference between young and aged mice in E55+MuLV clearance is associated with, and possibly due to, age-related alterations in both T and B cell responses to the virus.

RESULTS

Clearance of E55+MuLV in 6- and 22-month-old B6 mice

Although previous studies in this laboratory have demonstrated that 8-week-old B6 mice inoculated with E55+MuLV were able to decrease virus burden to undetectable levels by 8 weeks p.i. (Panoutsakopoulou *et al.,* 1998), the effect of age on control of E55+MuLV had not been examined. We therefore assessed the ability of 6- (young) and 22- (aged) month-old B6 mice to decrease virus burden during the acute phase of E55+MuLV infection. Mice were inoculated with E55+MuLV and splenic virus titers were determined by fluorescent focus assay (FFA) at various times p.i. Both age groups expressed comparable levels of virus at 2 and 4 weeks p.i. (Table 1). However, by 8 weeks p.i., young mice decreased virus burden to undetectable levels, while aged mice continued to express high levels of virus in spleen. While virus titers were still detectable at 12 (Table 1) and 16 weeks p.i. (data not shown), aged mice finally decreased virus burden to undetectable levels by 20 weeks p.i. These data suggest that while 6-month-old mice expressed an infection profile similar to that of 8-week-old mice, the ability of 22-month-old mice to clear E55+MuLV was delayed considerably.

T cell proliferative and cytotoxic responses to E55+MuLV of young and aged B6 mice

To determine whether the observed difference in virus clearance between the two age groups was due to an age-associated alteration in the adaptive immune system, spleen cells from young and aged B6 mice infected with E55+MuLV were used in proliferation and cytotoxic T lymphocyte (CTL) assays at various times p.i. Splenocytes of young mice generated a significant proliferative response to E55+MuLV at 2 and 4 weeks p.i. that was maintained at 8 and 12 weeks p.i. (Fig. 1). In contrast, splenocytes of aged mice failed to generate a significant proliferative response to E55+MuLV at any time examined. $CD4^+$ T cells were required to obtain a significant proliferative response in young mice because *in vitro* depletion of these cells prior to stimulation completely abolished this response (data not shown). Depletion of $CD8^+$ T cells prior to stimulation decreased the level of responses by 10–20%, which was not a statistically significant reduction (data not shown).

Virus-specific CTL activity was first detected in both

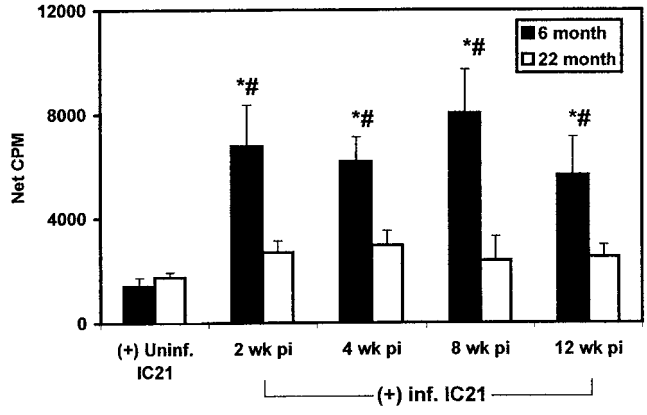

FIG. 1. T cell proliferative response to E55+MuLV in young and aged mice. Spleen cells from individual naive or E55+MuLV-infected B6 mice, at 2, 4, 8, and 12 weeks p.i. were cultured with either naive or E55+MuLV-infected IC21 cells. Proliferation is expressed as net counts per minute (Net CPM). Values are means ± SEM of 6–12 individual mice assayed in three separate experiments, 2–4 mice/group/experiment. Values of the control group (spleen cells cultured with uninfected IC21 cells) are means ± SEM of all time points tested. Statistically significant differences as determined by Student's *t* test; * naive vs infected IC21 cells, $P < 0.02$; # young vs aged of same time point, $P < 0.05$ ANOVA analysis of all time points of one age group demonstrated no statistically significant differences.

age groups at 3 weeks p.i. (Fig. 2A), with the peak virus-specific CTL activity being detected at 4 weeks p.i. (Fig. 2B). By 8 weeks p.i. the CTL response was undetectable in both age groups and remained undetectable at 12 weeks (data not shown). Although the kinetics of virus-specific CTL response were comparable in both age groups, the CTL response was consistently and significantly higher in young mice ($P < 0.04$). $CD8^+$ T cells mediated cytotoxicity in both age groups because *in vitro* depletion of these cells prior to assay completely abrogated the CTL response (Fig. 2B).

Role of B cells in clearance of E55+MuLV in young mice

B cells play a crucial role in the clearance of some primary virus infections (Bachmann and Kopf, 1999). While B-cell-deficient mice (μMT) showed decreased resistance to Friend virus (FV)-induced splenomegaly, suggesting that B cells are required for the control of FV infection (Hasenkrug, 1999), the role of B cells in control of E55+MuLV infection was unknown. To address this question, 8-week-old μMT mice were inoculated with E55+MuLV and splenic virus titers were determined by FFA at 8 weeks p.i. Unlike intact B6 mice, μMT mice were unable to lower the virus burden by 8 weeks p.i. even after inoculation with a 10-fold lower dose of E55+MuLV (Table 2). In fact, the μMT mice expressed virus levels comparable to virus titers in irradiated B6 mice. These results suggest that B cells play a role in decreasing virus burden by 8 weeks p.i. in young B6 mice.

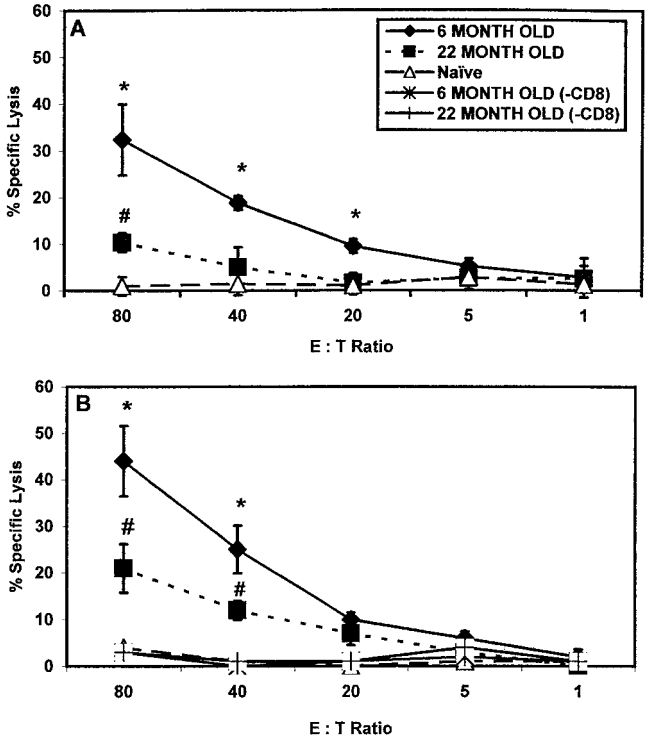

FIG. 2. E55+MuLV-specific cytotoxic T cell response of young and aged B6 mice. Pooled spleen cells from 3–4 naive (△) or E55+MuLV-infected young (◆) and aged (■) B6 mice at 3 (A) and 4 (B) weeks p.i. were used as effector cells in a CTL assay against uninfected and virus-infected IC21 cells ($H\text{-}2^b$). Aliquots of spleen cells were depleted of $CD8^+$ T cells prior to assay (x, +). Cytotoxicity of both KKC ($H\text{-}2^k$) and uninfected IC21 cells was ≤5% at all E:T ratios. The data shown are means ± SEM of three separate experiments. Statistically significant differences as determined by Student's *t* test; * young vs aged, $P < 0.04$, # aged vs naive, $P < 0.02$.

TABLE 2

E55+ Virus Production in μMT Mice[a]

Dose of virus (FFU)	Recipient mice: C57BL/6	Irradiated C57BL/6 (450 rad/mouse)	C57BL/6-Igh (μMT)
4×10^4	<10	3×10^2	2×10^2
	<10	2×10^2	2×10^3
	<10	3×10^2	3×10^1
4×10^3	<10	3×10^1	1×10^3
	<10	1×10^2	1×10^2
	<10	4×10^1	1×10^2

[a] Eight-week-old mice were infected with either the standard dose or a 10-fold lower dose of E55+MuLV. All mice were sacrificed at 8 weeks p.i. and virus titers were determined by FFA. Titers were expressed as FFU/ml of 10% spleen homogenate.

Virus-neutralizing antibody titers in young and aged B6 mice

Since our data suggested that B cells are required for decreasing virus burden by 8 weeks p.i. in young mice, we measured the E55+MuLV-specific neutralizing antibody (Ab) titers in sera of young and aged B6 mice at various times p.i. All B6 mice were pre-bled, inoculated with E55+MuLV, and bled again at 2, 4, 8, or 12 weeks p.i. The sera were then evaluated by virus neutralization assay. The results show that at 2 weeks p.i., 8 of 10 young mice and only 1 of 10 aged mice had detectable Ab titers (Fig. 3). At 4 weeks p.i. all young mice tested (15 mice) had detectable Ab titers; 15 of 17 aged mice had detectable Ab titers. Although mean Ab titers were significantly higher at 4 weeks p.i. than at 2 weeks p.i. in both age groups ($P < 0.0005$), young mice had significantly higher mean Ab titers than aged mice at 4 weeks p.i. ($P < 0.02$; Fig. 3). All mice tested at 8 weeks p.i. (12 mice/age group) had detectable Ab titers, but young mice still had significantly higher mean Ab titers than aged mice. Similar results were obtained at 12 weeks p.i., except that only 7 of 9 aged mice tested had detectable Ab titers while all young mice (9 mice) had detectable Ab titers. Collectively, these results indicate that aged mice demonstrate both a delay in the generation of virus-neutralizing antibodies and significantly lower mean Ab titers compared to young B6 mice.

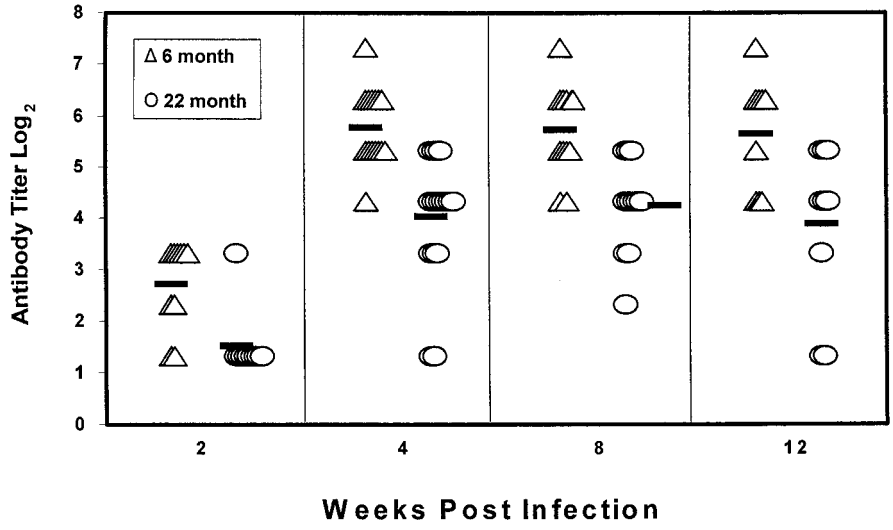

FIG. 3. E55+MuLV neutralizing antibody titers of young and aged B6 mice. Young and aged B6 mice were infected with E55+MuLV. Antibody titers were determined for individual mice at 2, 4, 8, and 12 weeks p.i. Data are from three separate experiments (3–6 mice/group/experiment). Bars represent the mean $\log_2$ titer/group. Statistically significant differences as determined by Student's *t* test: young vs aged, $P < 0.02$ at all times; 2 weeks vs 4 weeks p.i., $P < 0.0005$ in both age groups.

Although antibody was determined to be required for control of E55+MuLV infection in young μMT (Table 2), antibody alone did not seem to be sufficient for control of E55+MuLV in aged mice. Five aged mice were assessed for both virus titers and neutralizing antibody titers at 12 weeks postinfection. While all five mice had detectable virus titers (10^2–10^4 focus-forming units (FFU)/ml), two of the five mice had high antibody titers, one had low antibody, and two had no detectable antibody. Of the two mice with virus titers of 10^4, one had no antibody while one had high antibody (data not shown).

DISCUSSION

The increased incidence and severity of virus infections that occur with increasing age have been attributed to an age-associated alteration(s) in specific immune responses (Schneider, 1983; Louria *et al.,* 1993). While many studies have demonstrated that aging is associated with alterations in T and B cell function (Kirschmann and Murasko, 1992; Goonewardene and Murasko, 1994; Chakravarti and Abraham, 1999; Pawelec *et al.,* 1999; LeMaoult *et al.,* 1997; Song *et al.,* 1997), only a limited number of studies have examined the association between decreased immune responsiveness and diminished control of primary virus infections in the aged (Jacoby *et al.,* 1994; Bender *et al.,* 1995). In the current studies we have characterized a model that could be used to investigate further this relationship.

Previous studies in this laboratory have demonstrated that E55+MuLV infection of young B6 mice is characterized by the presence of large numbers of virus-infected cells in the spleen and bone marrow during the first 2–4 weeks p.i. The generation of an anti-virus immune response plays a crucial role in decreasing virus burden to undetectable levels by 8 weeks p.i. (Panoutsakopoulou *et al.,* 1998; Avidan *et al.,* 1995). We hypothesized that an age-associated decrease in the immune response against E55+MuLV would lead to failure of the aged mice to decrease virus burden by 8 weeks p.i. Our results clearly show that the spleens of aged B6 mice continued to express detectable levels of E55+MuLV at 8 and 12 weeks p.i., while virus was undetectable in young B6 mice. Although we expected virus titers to persist throughout the remaining life span of aged mice, we found that aged mice were able to decrease virus burden to undetectable levels by 20 weeks p.i. This delay in the ability of aged mice to clear E55+MuLV is similar to results with influenza virus. Unlike young mice that stopped shedding virus by 7–8 days p.i., aged mice continued to retain virus in their lungs for 12–13 days p.i. (Bender *et al.,* 1991, 1995). These results are consistent with the observation that elderly individuals have a prolonged duration of illness following viral infections.

$CD4^+$ T cells play an important role in the control of some virus infections. In the closely related FV system, *in vivo* depletion of $CD4^+$ T cells prior to infection resulted in a dramatic increase in splenomegaly and mortality of FV-infected mice by 60–90 days p.i. (Robertson *et al.,* 1992). Similarly, *in vivo* depletion of $CD4^+$ T cells prior to mouse poxvirus (MPV) infection resulted in a significant increase in splenic and liver virus titers and incomplete virus clearance (Karupiah *et al.,* 1996). Previous studies in this laboratory have demonstrated the importance of $CD4^+$ T cells in the control of E55+MuLV infection in young mice. *In vivo* $CD4^+$ T cell depletion prior to E55+MuLV inoculation of young B6 mice completely

abrogated their ability to decrease virus burden during the acute phase of infection (Panoutsakopoulou *et al.,* 1998). In this system, $CD4^+$ T cells were able to mediate reduction of virus burden in the absence of $CD8^+$ T cells since *in vivo* $CD8^+$ T cell depletion did not affect the ability of the 8-week-old mice to decrease virus burden (Panoutsakopoulou *et al.,* 1998).

In the current studies, the proliferative response of T cells to E55+MuLV was examined in young and aged mice. Both young and aged mice expressed comparable levels of virus in spleen at 2 and 4 weeks p.i. However, only splenocytes of young mice generated a significant proliferative response to E55+MuLV. This response was dependent on $CD4^+$ T cells since depletion of CD4 cells prior to culture abrogated all reactivity, while depletion of CD8 cells prior to culture reduced the proliferative response only 10–20%. Importantly, the proliferative response of young mice was detected as early as 2 weeks p.i. and was maintained at 12 weeks p.i., a time when the virus had been reduced. In contrast, aged mice never achieved a significant proliferative response to E55+MuLV at any of the times tested.

CTL activity plays a key role in the clearance of many virus infections. For example, *in vivo* depletion of $CD8^+$ T cells during primary simian immunodeficiency virus infection resulted in higher plasma viral RNA levels and higher levels of virus replication compared to the control group (Schmitz *et al.,* 1999). Similarly, *in vivo* depletion of $CD8^+$ T cells prior to FV infection resulted in 75% of mice dying or developing splenomegaly by 60–90 days p.i. compared to <10% of control mice (Robertson *et al.,* 1992). Depletion of $CD8^+$ T cells prior to infection with a dose of MPV that was nonlethal in intact mice resulted in 100% mortality (Karupiah *et al.,* 1996). Furthermore, influenza virus infection of mice lacking class I major histocompatibility complex (MHC)-restricted T cells (β_2-$M^{-/-}$) (Bender *et al.,* 1992) or of μMT mice depleted of $CD4^+$ T cells (Riberdy *et al.,* 2000) has indicated that $CD8^+$ T cells were necessary, and in some situations sufficient, for termination of influenza virus infection.

Based on this evidence, we examined E55+MuLV-specific CTL activity. The kinetics of E55+MuLV-specific CTL activity was similar in both young and aged mice. Although virus-specific CTL activity was not detectable at 2 weeks p.i., CTL activity increased by 3 weeks p.i., peaked at 4 weeks p.i., and declined to undetectable levels by 8 weeks p.i. CTL activity, however, was significantly higher in young mice than in aged mice at all time points ($P < 0.04$). Previous studies using the influenza virus model found that prolonged virus shedding in aged mice correlated with a 5- to 7-day delay in the generation of splenic anti-influenza CTL (Bender *et al.,* 1991) and a 1-day delay in the generation of pulmonary anti-influenza CTL activity (Bender *et al.,* 1995). While the delay in influenza virus clearance in aged mice appeared to be associated with a delay in generation of CTL activity, the delay in clearance of E55+MuLV may depend on an age-associated shift in the mechanism of immune control of virus infection. In young mice, $CD4^+$ T cells can mediate reduction in E55+MuLV titers in the absence of $CD8^+$ T cells (Panoutsakopoulou *et al.,* 1998). Since aged mice demonstrate no statistically significant $CD4^+$ T cell reactivity *in vitro* as assayed by proliferation, the eventual reduction in E55+MuLV titers may require $CD8^+$ T cells with little or no assistance from $CD4^+$ T cells.

The decline in E55+MuLV-specific CTL activity in aged mice may be due to an alteration in intrinsic $CD8^+$ T cell function. Alternatively, the observed decrease in E55+MuLV-specific CTL activity in aged mice may be due to age-related changes in the $CD4^+$ T-helper cells that may provide immunologic support for CTL. Reports on the requirements for $CD4^+$ T cell help for the generation of class I MHC-restricted antiviral CTL responses are controversial. Impaired CTL responses to vesicular stomatitis virus (VSV) and lymphocytic choriomeningitis virus (LCMV) occurred in mice depleted *in vivo* of $CD4^+$ T cells prior to virus inoculation (Leist *et al.,* 1989). Additional studies using MHC class II-deficient mice have found that VSV-specific primary CTL response was absent, while LCMV-specific primary CTL response was reduced (Battegay *et al.,* 1996) in infected mice. Similarly, *in vivo* depletion of $CD4^+$ T cells prior to MPV inoculation resulted in the generation of suboptimal virus-specific CTL at day 8 p.i. compared to control monoclonal antibody (mAb) treated mice (Karupiah *et al.,* 1996). In contrast, other studies using similar antibody depletion approaches have shown that $CD4^+$ T cells were not required for the generation of CTL responses against MPV (Buller *et al.,* 1987), LCMV (Ahmed *et al.,* 1988), and influenza (Riberdy *et al.,* 2000). Since our results showed an alteration in $CD4^+$ T cell function in aged mice, the possibility remains that the observed decrease in CTL activity in aged mice may have been due to age-related decline in both $CD4^+$ and $CD8^+$ T cell functions.

B cells also play a crucial role in the clearance of some viral infections. Polyoma virus (PyV) infection of SCID mice, which lack functional B and T cells, resulted in fatal acute myeloproliferative disease within 14–16 days p.i. in 100% of mice. In contrast, PyV infection of T-cell-deficient mice (TCR $\alpha\beta/\gamma\delta$ $-/-$), which had targeted disruption of their TCR α and β genes, resulted in 100% survival, regardless of the presence or absence of NK cells (Szomolanyi-Tsuda and Welsh, 1996). In another study, athymic nude mice and TCR $\alpha\beta/\gamma\delta$ $-/-$ mice were able to clear primary rotavirus infection, while only 40% of SCID mice cleared all intestinal virus (Franco and Greenberg, 1997). This study suggests that B cells, possibly with some innate immune mechanisms, can clear rotavirus. In some systems, B cells were found to be required for control of virus infections only in specific situations. For example, in mice resistant to FV infection

due to the FV-2 gene, B cell deficiency (μMT $-/-$) had only a limited effect on splenomegaly (Hasenkrug, 1999), while depletion of B cells in susceptible mice always results in the development of erythroleukemia (Doig and Cheeseboro, 1979). Similarly, μMT mice were more susceptible to influenza as demonstrated by a 50- to 100-fold reduction in the LD_{50} (Graham and Braciale, 1997). However, when μMT mice are infected with a dose of virus that is nonlethal, virus clearance occurs and is dependent on $CD8^+$ T cells (Riberly *et al.,* 2000).

We utilized μMT mice to examine the role of B cells in the control of E55+MuLV infection in young B6 mice. Young μMT mice were unable to decrease E55+MuLV burden by 8 weeks p.i., even after inoculation with a 10-fold lower dose of virus. They expressed virus levels comparable to virus titers of irradiated–infected B6 mice. Since these results suggested that B cells are required for early clearance of E55+MuLV in young mice, we determined the differences in virus-neutralizing Ab titers between young and aged B6 mice at various times p.i. A delay in the generation of virus-neutralizing Ab was observed in aged mice. In addition, young mice had significantly higher Ab titers than aged mice at all times tested. These results are consistent with other observations that show that the antibody response to T-cell-dependent foreign antigens decreases dramatically with age (Goidl *et al.,* 1976).

The age-related decrease in anti-E55+MuLV antibody titers may be due to an intrinsic alteration in B cell function. Alternatively, since activated $CD4^+$ T-helper cells provide immunologic support for B cells, and our results suggest an alteration in $CD4^+$ T cell function in aged mice, the observed decrease in virus-neutralizing Ab titers in aged mice may have occurred because the aged $CD4^+$ T cells provided less efficient help to the B cells. In young FV-infected mice, $CD4^+$ T cells are required for production of virus-neutralizing antibodies since $CD4^+$ T-cell-depleted mice lacked detectable FV-neutralizing antibodies at 30 days p.i. (Super *et al.,* 1998). Similarly, in other studies using mice transgenic for the human poliovirus receptor, the adoptive transfer of both poliovirus-specific $CD4^+$ T and B cells, but not either cell type alone, was required to confer protection following challenge with a virulent strain of poliovirus (Mahon *et al.,* 1995).

In summary, E55+MuLV provides a unique model by which age-associated changes in the anti-virus immune response can be studied. The long acute phase of virus expression allows careful evaluation of the role of alterations in the kinetics of immune response on control of virus. The reexpression of E55+MuLV in persistently infected susceptible mice allows the examination of the role of the level and type of immune response required for maintenance of this control. Lack of reexpression of E55+MuLV in persistently infected aged mice of the resistant strain would suggest that control requires a fairly limited immune response. In addition, following infected young mice of the resistant strain for a longer period of time will determine whether the natural decline in immune responses that occurs with aging will result in reemergence of the virus and progression to leukemia. We are currently utilizing this model to assess differences in the cytokine profiles of young and aged mice during the acute phase of E55+MuLV infection. We will also determine whether adoptive transfer of cells from young mice can alter the pattern of E55+MuLV clearance in aged mice.

MATERIALS AND METHODS

Mice

Six- and 22-month-old C57BL/6 (B6) male mice were purchased from the NIA colony of Charles River Laboratories. Eight-week-old μMT (C57BL/6-Igh-^{6tm1Cgn}) and B6 mice were purchased from The Jackson Laboratories (Bar Harbor, ME). All mice were housed in AAALAC-certified barrier facilities at MCP Hahnemann University and were given autoclaved food and water *ad libitum.* Mice were allowed to acclimate for at least 2 weeks in our facilities prior to use. Mice demonstrating tumors of any kind were eliminated from the study.

Virus

E55+ murine leukemia virus was originally isolated from a BALB.K spleen injected with cell-free culture supernatant from a T cell line derived from a leukemic mouse (Pozsgay *et al.,* 1989). The virus used in these studies was propagated *in vivo* by intraperitoneal (ip) injections of immunosuppressed BALB/c mice (Wolf and Blank, 1983). All mice used in the studies were injected ip with 4×10^4 FFU of E55+MuLV, unless indicated otherwise.

Antibodies

Hybridoma cells producing the monoclonal antibodies mAb34, specific for the p15-*gag* protein, and mAb48, specific for the gp70 envelope protein (Cheseboro *et al.,* 1981), were a gift from Dr. Bruce Cheseboro. Hybridoma cells producing the mAb GK1.5 (anti-CD4) and 2.43 (anti-CD8) were obtained from American Type Culture Collection (ATCC; Rockville, MD). Antibodies were purified using protein A–Sepharose affinity chromatography (Ey *et al.,* 1978).

Cells

The IC21 cell line is an SV40-transformed macrophage line derived from peritoneal macrophages of normal B6 mice and was obtained from ATCC. IC21 cells express class I ($H\text{-}2^b$) and class II ($I\text{-}A^b$) MHC molecules. E55+MuLV-infected IC21 cells express viral epitopes of the *env* and *gag* gene products, as determined by flow

cytometric staining with mAb34 and mAb48. The KKC tumor cell line (H-2^k, class I positive and class II negative) was established in our laboratory from the leukemic spleen of an immunosuppressed (550 rad) BALB/c-*H*-2^k (BALB.K) mouse inoculated with E55+MuLV. KKC and IC21 cells were maintained in RPMI 1640 (Media Tech, Herndon, VA) with 10% FCS (Sigma, St. Louis, MO), 1 mM L-glutamate, 100 U penicillin, 0.1 mg streptomycin, and 5×10^{-5} M 2-mercaptoethanol (complete medium). The *Mus dunni* fibroblast cell line was a gift from Dr. Harvey Schlesinger and was maintained in DMEM (Media Tech) with 10% FCS, 1 mM L-glutamate, 100 U penicillin, and 0.1 mg streptomycin (maintenance medium).

Fluorescent focus assay

FFA was performed as previously described (Sitbon *et al.,* 1985). Briefly, *M. dunni* cells were plated at 6×10^3 cells/well in 24-well plates in maintenance medium with 10 μg polybrene/ml (Sigma). Cells were infected with serial 10-fold dilutions of spleen homogenates. One hour after infection, wells were washed with PBS and fresh maintenance medium was added. Cells were cultured for 3–4 days until confluent. Cells were washed with PBS, stained with mAb34 for 1 h at 4°C, and then stained with FITC-conjugated anti-mouse Ig antibody (Southern Biotechnology, Birmingham, AL) for 1 h at 4°C. After being washed with PBS, the fluorescent foci were counted using an inverted fluorescence microscope (Zeiss-IM, Germany). Virus titers were expressed as fluorescent FFU per milliliter of spleen homogenate. Although the FFA can detect 1 FFU/ml, the spleens were a 10% homogenate and, therefore, the sensitivity of this assay is 10 FFU/ml of homogenate.

T cell proliferation assay

Spleen cells from individual naive or E55+MuLV-infected B6 mice were harvested at various times starting at 2 weeks p.i. and were cultured in complete medium in 96-well plates at 1×10^5 cells/well in the presence of 2.5×10^4 naive or E55+MuLV-infected IC21 cells. IC21 cells were treated with mitomycin C (50 ng/ml) for 1 h at 37°C and then washed 3–4 times with PBS before use. Splenocytes were cultured for 72 h and pulsed with 1.0 μCi [^{3}H]thymidine per well during the final 12 h of culture. Cells were harvested using a PHD cell harvester (Cambridge Technologies, Cambridge, MA) and radioactivity was counted in a liquid scintillation counter (Beckman, Columbia, MD). Proliferation is expressed as net counts per minute (cpm) calculated as:

$$\text{Net cpm} = (\text{cpm of splenocytes with IC21 cells}) - (\text{cpm of splenocytes alone}).$$

Cell-mediated cytotoxicity assay

Pooled spleen cells from three or four naive or E55+MuLV-infected B6 mice were harvested at various times starting at 2 weeks p.i. and were cultured in 24-well plates at 8×10^6 cells/well with 1×10^6 E55+MuLV-infected IC21 cells. Virus-infected IC21 cells were treated with mitomycin as described above. Mixtures were cultured for 5 days in complete medium at 37°C in 5% CO_2. Cells were then washed, counted, resuspended in complete medium, and used as effectors in the JAM assay (Matzinger, 1991). Briefly, target cells (naive or E55+MuLV-infected IC21 cells, or KKC cells) were pulsed with 3–4 μCi [^{3}H]thymidine for 6 h, washed, and then plated at 1×10^4 cells/well in round-bottom 96-well plates. Effectors and targets were incubated at various E:T ratios for 4 h and then harvested using a PHD cell harvester. Retained radioactivity was counted in a liquid scintillation counter. In some cases, the effector cells were depleted *in vitro* of $CD8^+$ T cells with antibody and complement before addition to the target cells as previously described (Panoutsakopoulou *et al.,* 1998). Percentage specific lysis was calculated as

$$\text{Percentage Lysis} = \{[(S - E)/S] \times 100\},$$

where S (spontaneous) is retained ^{3}H in the absence of effectors and E (experimental) is retained ^{3}H in the presence of effectors. Cytotoxicity of both uninfected IC21 and H-2 incompatible KKC (H-2^k) target cells by effector cells from E55+MuLV-infected mice was consistently $\leq$5% at all E:T ratios.

Virus neutralization assay

Serial twofold dilutions of sera from individual naive or E55+MuLV-infected mice were incubated with 5×10^2 FFU of E55+MuLV for 1 h at 37°C in 5% CO_2. As negative controls, virus was incubated either with medium alone or with serial dilutions of immune sera from herpes simplex virus-infected mice. The serum–virus mixture was then added to previously plated *M. dunni* cells in 24-well plates and FFA was performed as described above. The virus-neutralizing antibody titer was defined as the reciprocal of the highest dilution of serum that completely inhibited E55+MuLV infection of *M. dunni* cells. As a positive control, anti-gp 70 polyclonal antibody (Quality Biotech Inc., Camden, NJ) was used in every assay and consistently had an antibody titer of 1:160. All results are presented as $\log_2$ of the titer.

Statistical analysis

Data are presented as means ± SEM. All statistics were performed using JMP software version 3.2.6 (SAS Institute). Statistical significance between groups was determined using either unpaired Student's *t* test or an ANOVA.

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REFERENCES

Adler, W. H., and Nagel, J. E. (1994). Acquired immunodeficiency syndrome in the elderly. *Drugs Aging* **4,** 410–416.

Adler, W. H., Baskar, P. V., Chrest, F. J., Dorsey-Cooper, B., Winchurch, R. A., and Nagel, J. E. (1997). HIV infection and aging: Mechanism to explain the accelerated rate of progression in the older patient. *Mech. Aging Dev.* **96,** 137–155.

Ahmed, R., Buttler, L. D., and Bhatti, L. (1988). T4+ T helper cell function in vivo: Differential requirement for induction of antiviral cytotoxic T cell and antibody responses. *J. Virol.* **62,** 2102–2106.

Avidan, N., Tumas-Brundage, K. M., Sieck, T. G., Prystowsky, M. B., and Blank, K. J. (1995). Effect of non-H-2-linked genes on anti-virus immune responses and long-term survival in mice persistently infected with E55+ murine leukemia virus. *Virology* **211,** 507–515.

Bachmann, M. F., and Kopf, M. (1999). The role of B cell in acute and chronic infections. *Curr. Opin. Immunol.* **11,** 332–339.

Battegay, M., Bachmann, M. F., Burhkart, C., Viville, S., Benoist, C., Mathis, D., Hengartner, H., and Zinkernagel, R. M. (1996). Antiviral immune responses of mice lacking MHC II or its associate invariant chain. *Cell. Immunol.* **167,** 115–121.

Bender, B. S., Johnson, M. P., and Small, P. A. (1991). Influenza in senescent mice: Impaired cytotoxic T-lymphocyte activity is correlated with prolonged infection. *Immunology* **72,** 154–159.

Bender, B. S., Croghan, T., Zhang, L., and Small, P. A. (1992). Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. *J. Exp. Med.* **175,** 1143–1145.

Bender, B. S., Taylor, S. T., Zander, D. S., and Cottey, R. (1995). Pulmonary immune response of young and aged mice after influenza challenge. *J. Lab. Clin. Med.* **126,** 169–177.

Buller, R. M. L., Holmes, K. L., Hugin, A., Frederickson, T. N., and Morse, H. C., III (1987). Induction of cytotoxic T cell responses in vivo in the absence of $CD4^+$ T cells. *Nature* **328,** 77–79.

Chakravarti, B., and Abraham, G. N. (1999). Aging and T cell-mediated immunity. *Mech. Aging Dev.* **108,** 183–206.

Cheseboro, B., Wehrly, K., Cloyd, M., Britt, W., Portis, J., Collins, J., and Nishio, J. (1981). Characterization of mouse monoclonal antibodies specific for Friend murine leukemia virus-induced erythroleukemia cells: Friend-specific and FMR-specific antigens. *Virology* **112,** 131–144.

Doig, D., and Chesebro, B. (1979). Anti-Friend virus antibody is associated with recovery from viremia and loss of viral leukemia cell surface antigens in leukemic mice: Identification of Rfv-3 as a gene locus influencing antibody production. *J. Exp. Med.* **150,** 10–19.

Ey, P. L., Prowse, S. J., and Jenkin, C. R. (1978). Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulins from mouse serum using protein A–Sepharose. *Immunochemistry* **15,** 429–436.

Felser, J. M., and Raff, M. J. (1983). Infectious diseases and aging: Immunologic perspectives. *J. Am. Geriatrics Soc.* **31,** 802–807.

Franco, M. A., and Greenberg, H. B. (1997). Immunity to rotavirus in T cell deficient mice. *Virology* **238,** 169–179.

Goidl, E. A., Innes, J. B., and Weksler, M. E. (1976). Loss of high avidity plaque-forming cells and increased suppressor cell activity in aging mice. *J. Exp. Med.* **144,** 1037–1048.

Goonewardene, I. M., and Murasko, D. M. (1994). Age associated changes in mitogen induced proliferation and cytokine production by lymphocytes of the long-lived brown Norway rat. *Mech. Aging Dev.* **71,** 199–212.

Graham, M. B., and Braciale, T. (1997). Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. *J. Exp. Med.* **186,** 2063–2068.

Hasenkrug, K. J. (1999). Lymphocyte deficiencies increase susceptibility to Friend virus-induced erythroleukemia in *Fv-2* genetically resistant mice. *J. Virol.* **73,** 6468–6473.

Jacoby, R. O., Bhatt, P. N., Barthold, S. W., and Brownstein, D. G. (1994). Sendai viral pneumonia in aged BALB/c mice. *Exp. Gerontol.* **29,** 89–100.

Karupiah, G., Buller, R. M. L., Van Rooijen, N., Duarte, C. J., and Chen, J. (1996). Different roles for $CD4^+$ and $CD8^+$ T lymphocytes and macrophage subsets in the control of a generalized virus infection. *J. Virol.* **70,** 301–309.

Kirschmann, D. A., and Murasko, D. M. (1992). Effect of endogenous cytokines on the inhibition of macrophage-induced, antigen-specific T cell proliferation by poly I:C. *Clin. Immunol. Immunopathol.* **3,** 300–307.

Leist, T. P., Kohler, M., and Zinkernagel, R. M. (1989). Impaired generation of anti-viral cytotoxicity against lymphocytic choriomeningitis and vaccinia virus in mice treated with CD4-specific monoclonal antibody. *Scand. J. Immunol.* **30,** 679–686.

LeMaoult, J., Szabo, P., and Weksler, M. E. (1997). Effects of age on humoral immunity, selection of the B-cell repertoire and B-cell development. *Immunol. Rev.* **160,** 115–126.

Louria, D. B., Sen, P., Sherer, C., and Farrer, W. E. (1993). Infections in older patients: A systemic clinical approach. *Geriatrics* **48,** 28–34.

Mahon, B. P., Katrak, K., Nomoto, A., Macadam, A. J., Minor, P. D., and Mills, K. H. (1995). Poliovirus-specific $CD4^+$ Th1 clones with both cytotoxic and helper activity mediate protective humoral immunity against a lethal poliovirus infection in transgenic mice expressing the human poliovirus receptor. *J. Exp. Med.* **181,** 1285–1292.

Matzinger, P. (1991). The JAM test. *J. Immunol. Methods* **145,** 185–192.

Panoutsakopoulou, V., Little, C. S., Sieck, T. G., Blankenhorn, E. P., and Blank, K. J. (1998). Differences in the immune response during the acute phase of E55+ murine leukemia virus infection in progressor BALB and long term nonprogressor C57BL mice. *J. Immunol.* **161,** 17–26.

Pawelec, G., Wagner, W., Adibzadeh, M., and Engel, A. (1999). T cell immunosenescence in vitro and in vivo. *Exp. Gerontol.* **34,** 419–429.

Pozsgay, J. M., Klyczek, K. K., and Blank, K. J. (1989). *In vivo* generation of antigenic variants of murine retroviruses. *Virology* **173,** 330–334.

Riberdy, J. M., Christensen, J. P., Branum, K., and Doherty, P. C. (2000). Diminished primary and secondary influenza virus-specific $CD8^+$ T-cell responses in CD4-depleted $Ig^{-/-}$ mice. *J. Virol.* **74,** 9762–9765.

Robertson, M. N., Spangrude, G. J., Hasenkrug, K., Perry, L., Nishio, J., Wehrly, K., and Chesebro, B. (1992). Role and specificity of T-cell subsets in spontaneous recovery from Friend virus-induced leukemia in mice. *J. Virol.* **66,** 3271–3277.

Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., Montefiori, D. C., Rieber, E. P., Letvin, N. L., and Reimann, K. A. (1999). Control of viremia in simian immunodeficiency virus infection by $CD8^+$ lymphocytes. *Science* **283,** 857–860.

Schneider, E. L. (1983). Infectious diseases in the elderly. *Ann. Int. Med.* **98,** 395–400.

Ship, J. A., Wolff, A., and Selik, R. M. (1991). Epidemiology of acquired immune deficiency syndrome in persons aged 50 years or older. *J. Acquired Immune Defic. Syndr.* **4,** 84–88.

Sitbon, M., Nishio, J., Wehrly, K., Lodmell, D., and Cheseboro, B. (1985). Use of a focal immunofluorescence assay on live cells for quantitation of retroviruses: Distinction of host range classes in virus mixtures and biological cloning of dual-tropic murine leukemia viruses. *Virology* **141,** 110–118.

Song, H., Price, P. W., and Cerny, J. (1997). Age-related changes in antibody repertoire: Contribution from T cells. *Immunol. Rev.* **160,** 55–62.

Super, H. J., Brooks, D., Hasenkrug, K., and Chesebro, B. (1998). Re-

quirement for $CD4^+$ T cells in Friend murine retrovirus neutralizing antibody response: Evidence for functional T cells in genetic low-recovery mice. *J. Virol.* **72,** 9400–9403.

Szomolanyi-Tsuda, E., and Welsh, R. M. (1996). T cell-independent antibody mediated clearance of polyoma virus in T cell deficient mice. *J. Exp. Med.* **183,** 403–411.

Tumas, K. M., Pozsgay, J. M., Avidan, N., Ksiazek, S. J., Overmoyer, B., Blank, K. J., and Prystowsky, M. B. (1993a). Loss of antigenic epitopes as the result of env gene recombination in retrovirus-induced leukemia in immunocompetent mice. *Virology* **192,** 587–595.

Tumas, K. M., Overmoyer, B., Clevenger, C. V., Blank, K. J., and Prystowsky, M. B. (1993b). Murine leukemia virus infection in immunocompetent adult mice. *Virology* **192,** 1–10.

Wolf, J. H., and Blank, K. J. (1983). Identification of a variant of gross leukemia virus that induces disease in mice inoculated as adults. *J. Exp. Med.* **158,** 629–634.